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Award Number: DAMD17-00-1-0390

TITLE: A Novel Phosphatase Gene on 10q23, *MINNP*, in Hereditary and Sporadic Breast Cancer

PRINCIPAL INVESTIGATOR: Charis Eng, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Ohio State University Research Foundation
Columbus, Ohio 43210-1063

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

PTEN is a tumor suppressor gene on 10q23 and encodes a dual specificity phosphatase. One of the major substrates for PTEN is phosphotidylinositol (3,4,5) triphosphate in the PI3 kinase pathway. When PTEN is dysfunctional or absent, P-Akt is high and hence, anti-apoptotic. *PTEN* is a major susceptibility gene for Cowden syndrome (CS), a hereditary disorder with a high risk of breast and thyroid cancer, and appears to be involved in a broad range of tumors. In addition, germline *PTEN*mutations have been found in a developmental disorder, Bannayan-Riley-Ruvalcaba syndrome (BRR) as well. Previously not thought to be associated with cancer risk, BRR families and cases with germline *PTEN* mutations have recently been shown to be at risk for cancers and especially breast tumors. Between 10-80% (mean 60%) of CS families and 60% of BRR individuals have germline *PTEN* mutations. Families that do not have germline *PTEN* mutations are not inconsistent with linkage to the 10q22-23 region. Thus, genes with related function to *PTEN* in the 10q21-q25 region are good candidates genes for *PTEN* mutation negative CS, BRR and related sporadic tumors, eg, those of the breast and thyroid. *MINPP1* lies no more than 1 Mb upstream of *PTEN* and encodes an inositol polyphosphate phosphatase. In Year 2 of the award, we have found no germline intragenic *MINPP1* mutations in 30 CS, 35 BRR and 15 CS-like. However, we have found at least 2 CS probands with germline deletions involving *MINPP1* and *PTEN*, and 1 CS with a deletion involving part of *PTEN*. In addition, at least 2 CS probands without *PTEN* or *MINPP1* alterations have been found to have germline *BMPR1A* mutations (this gene lies just upstream of *MINPP*). While we have not found somatic intragenic *MINPP1* mutations in 50 sporadic breast cancers, we have found somatic deletions encompassing *PTEN* and *MINPP1*. More interestingly these deletions involve the neoplastic epithelium and/or the surrounding stroma.

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Table of Contents

Year 2 Annual Progress Report

Proposal Title: A novel phosphatase gene on 10q23, *MINPP*, in hereditary and sporadic breast cancer (DAMD17-00-1-0390)

PI: Charis Eng, MD, PhD

INTRODUCTION

PTEN is a tumor suppressor gene on 10q23 and encodes a dual specificity phosphatase. One of the major substrates for PTEN is phosphotidylinositol (3,4,5) triphosphate in the PI3 kinase pathway. Downstream of this pathway lies Akt/PKB, a known cell survival factor. When PTEN is functional and abundant, Akt is hypophosphorylated and hence, pro-apoptotic. Conversely, when PTEN is dysfunctional or absent, P-Akt is high and hence, anti-apoptotic. *PTEN* is a major susceptibility gene for Cowden syndrome (CS), a hereditary disorder with a high risk of breast and thyroid cancer, and appears to be involved in a broad range of tumors. In addition, germline *PTEN*mutations have been found in a developmental disorder, Bannayan-Riley-Ruvalcaba syndrome (BRR) as well. This is an autosomal dominant disorder characterised by macrocephaly, lipomatosis, hemangiomatosis and speckled penis. Previously not thought to be associated with cancer risk, BRR families and cases with germline *PTEN* mutations have recently been shown to be at risk for cancers and especially breast tumors. Between 10-80% (mean 60%) of CS families and 60% of BRR individuals have germline *PTEN* mutations. Families that do not have germline *PTEN* mutations are not inconsistent with linkage to the 10q22-23 region. While breast cancer is a major component of CS and 30-50% of sporadic tumors carry hemizygous deletion in the 10q22-23 region, no or rare sporadic breast carcinomas have somatic intragenic *PTEN* mutations. A gene encoding a novel inositol polyphosphate phosphatase, *MINPP*, with overlapping function with PTEN, has been mapped to 10q23. We hypothesise that *MINPP* will be the susceptibility gene for the remainder of CS and BRR families and might likely be the major tumor suppressor gene on 10q23 which plays a role in the pathogenesis of sporadic breast carcinomas. We hope to explore whether *MINPP* is another CS and BRR susceptibility gene by looking for germline mutations in cases without germline *PTEN* mutations. We will also perform mutation and fine structure deletion analysis of *MINPP* in sporadic breast carcinomas. And finally, to prove that *MINPP* is a tumor suppressor and to begin to explore its relationship with PTEN in breast carcinogenesis, we will perform stable transfection experiments into two breast cancer lines with known genomic *PTEN* status (one PTEN wildtype and one PTEN null) as well as known PTEN protein and P-Akt levels. We will especially determine if MINPP is growth suppressive like PTEN, and determine if growth suppression is mediated by G1 arrest and/or apoptosis. Towards these ends, our specific aims were:

1. To determine if germline mutations of *MINPP* cause *PTEN* mutation negative CS, BRR and CS-like families.
2. To determine if somatic *MINPP* mutations and deletions are associated with sporadic breast carcinomas.
3. To determine if MINPP affects Akt activity and causes G1 arrest and/or cell death in breast cancer cell lines.

BODY

Task 1: Mutation analysis of *MINPP* in germline *PTEN* mutation negative CS, BRR and CS-like Cases

Thirty unrelated CS probands, 35 unrelated BRR probands and 15 unrelated Proteus syndrome probands known not to harbor germline *PTEN* mutations by PCR-based DGGE and sequencing have thus far been ascertained for this particular task. CS and BRR were diagnosed stringently by the criteria of the International Cowden Consortium (1) and as documented previously (2), respectively. The criteria for the diagnosis of a CS-like individual or family is as previously described (3). Preliminary mutation analysis of all exons, exon-intron junctions and flanking intronic sequences of *MINPP1* have been performed on all these probands using a combination of PCR-based DGGE and direct sequencing. Amongst these 80 probands, no intragenic *MINPP* germline mutations have been found to date.

We then came up with an extended hypothesis that while germline intragenic mutations in *MINPP1* are going to be rare in *PTEN* mutation negative (by PCR-based strategies) CS/BRR/CS-like, large germline deletions and rearrangements involving *MINPP1* might occur. To test this hypothesis, a comprehensive approach to look for hemizygous mutations in the 10q22-q23 region encompassing *MINPP1* and *PTEN* was instituted. This included examining for possible hemizygosity of 10 microsatellite markers spanning this region as well as using real-time PCR to look for true germline hemizygosity. Microsatellite analysis and real-time PCR using probes only to exons 3 and 5 of *PTEN* have been completed for all 80 probands. Complete analyses have been performed for 3 CS cases. Of these 3, 2 were found to carry germline deletions which encompass the entire *PTEN* and the entire *MINPP1* but not much further 5'. The remaining 1 has a deletion whose 3' limits are exon 8 of *PTEN* but the 5' extent is not entirely characterized although it most likely involves at least the 3' end of *MINPP1*. Real-time PCR using probes to each of the exons of *PTEN* and *MINPP1* will be on-going.

In the last year, another gene 5' of *MINPP1* called *BMPR1A* encoding a receptor for the bone morphogenetic proteins (BMPs) which belong to the TGFB superfamily was uncovered as a susceptibility gene for juvenile polyposis. We examined this gene in *PTEN* mutation negative CS and BRR and found 2 CS families with germline *BMPR1A* mutations (4) [see appendix] (Eng et al unpublished). These 2 CS families were particularly characterized by colonic features, which is relatively unusual for classic CS families. See attached reprint as well.

Task 2: Mutation and deletion analysis of *MINPP* in sporadic primary human breast carcinomas

To further understand the role of *MINPP* in sporadic counterparts of CS component cancers, we are accruing two series of sporadic tumors, primary adenocarcinomas of the breast and primary follicular thyroid neoplasias. Currently, we have accrued 60 breast cancers and have examined the first 50 for somatic *MINPP* mutations. To date, no

obvious pathogenic intragenic mutations have been found. Accrual of breast tumors and *MINPP* mutation analysis continues.

In this series of breast cancers, we have also begun to look for somatic deletions in the *MINPP1* and *PTEN* regions using LOH analysis. However, working from the hypothesis that LOH can occur equally in the neoplastic epithelium as well as the surrounding stroma, we analyzed 50 sporadic invasive ductal carcinomas for LOH at markers D10S581, D10S579, D10S1765 and D10S541 for LOH in epithelium and stroma, which have been separated by standard laser capture microdissection. Of the informative samples, 42% had LOH of at least 2 D10 markers in the epithelium alone 30% stroma alone and 15% in both compartments (5) [see appendix] (Eng et al, unpublished). See attached reprint as well. These preliminary observations are tantalizing and we will continue to pursue the somatic deletion angle. Further, we will also prepare to perform *MINPP1* and *PTEN* mutation analysis in the separate compartments.

Task 3: Functional studies of *MINPP* in *PTEN*+/+ and PTEN null breast cancer cell lines

MINPP1 cDNA constructs have now been made in pCR2.1 and in the mammalian expression system pUHD10-3 which contains a tetracycline-suppressible (Tet-off) promoter, as previously described for *PTEN* expression constructs (6). A single "pass" stable transfection in MCF-7 breast cancer lines (endogenous *PTEN* and *MINPP1* wildtype) did not produce any obvious growth suppressive effect. This will need to be repeated and other biochemical differences will need to be examined.

KEY RESEARCH ACCOMPLISHMENTS

- Germline *MINPP1* deletions, with or without accompanying *PTEN* deletion, may account for an unknown subset of *PTEN* mutation negative (by PCR) CS and BRR.

- *BMPR1A* might be a rare new susceptibility gene for *PTEN* and *MINPP* mutation negative CS and BRR, and might be associated with colonic phenotype.

- Somatic deletion of *MINPP1*, with or without accompanying deletion of *PTEN*, occurs in the neoplastic epithelium and/or surrounding stroma of sporadic invasive ductal carcinomas of the breast.

REPORTABLE OUTCOMES

Zhou XP, Woodford-Richens K, Lehtonen R, Kurose K, Aldred M, Hampel H, Launonen V, Virta S, Pilarski R, Salovaara R, Bodmer WF, Conrad BA, Dunlop M, Hodgson SV, Iwama T, Järvinen H, Kellokumpu I, Kim JC, Leggett B, Markie D, Mecklin J-P, Neale K, Phillips R, Piris J, Rozen P, Houlston R, Aaltonen LA, Tomlinson IPM, **Eng C**. Germline mutations in *BMPR1A/ALK3* cause a subset of juvenile polyposis syndrome and of Cowden and Bannayan-Riley-Ruvalcaba syndromes. Am J Hum Genet 2001; 69:704-11.

Kurose K, Hoshaw-Woodard S, Adeyinka A, Lemeshow S, Watson P, **Eng C**. Genetic model of multi-step breast carcinogenesis involving the epithelium and stroma: clues to tumour-microenvironment interactions. Hum Mol Genet 2001: 10:1907-13.

Conferred the William C. and Joan E. Davis Professorship of Cancer Research by The Ohio State University, Oct., 2001.

Promoted to Professor of Medicine, Human Cancer Genetics and Molecular Genetics, The Ohio State University, July 1, 2002.

CONCLUSIONS

The second year of work exploring MINPP as an alternative phosphatase in playing a role in the etiology and pathogenesis of CS and sporadic tumors has suggested interesting possibilities. Although we have found no intragenic germline *MINPP1* mutations in a total of 80 *PTEN* mutation negative CS, BRR and CS-like (Proteus. etc), the numbers per category are still relatively small, ie, sample size per category are not powered to detect eg ,5-10% prevalence of mutation. More interestingly, however. we have found that large germline *MINPP1* and *PTEN* mutations are associated with some subset of CS, BRR and CS-like. This will be an avenue that we will vigorously pursue in the final year of funding as this not only adds to our knowledge of phosphatases but it has clinical implications for such individuals and their families. Another exciting observation coming out of this second year of work is that somatic hemizygous deletion of *MINPP1* with or without accompanying deletion of *PTEN* can occur in the epithelium and/or the stroma of invasive ductal carcinomas of the breast. This observation suggests that genetic alterations in the stroma play some role in the pathogenesis of breast carcinomas and that there must be genetic cross talk between these two compartments. This avenue of work will be pursued in the final year of funding as well.

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6. Weng L-P, Smith WM, Dahia PLM, Ziebold U, Gil E, Lees JA, Eng C. PTEN suppresses breast cancer cell growth by phosphatase function-dependent G1 arrest followed by apoptosis. Cancer Res. 1999;59:5808-14.

APPENDIX

NIH-Style Biosketch

Two Reprints:

Zhou XP, Woodford-Richens K, Lehtonen R, Kurose K, Aldred M, Hampel H, Launonen V, Virta S, Pilarski R, Salovaara R, Bodmer WF, Conrad BA, Dunlop M, Hodgson SV, Iwama T, Järvinen H, Kellokumpu I, Kim JC, Leggett B, Markie D, Mecklin J-P, Neale K, Phillips R, Piris J, Rozen P, Houlston R, Aaltonen LA, Tomlinson IPM, Eng C. Germline mutations in *BMPR1A/ALK3* cause a subset of juvenile polyposis syndrome and of Cowden and Bannayan-Riley-Ruvalcaba syndromes. Am. J. Hum. Genet. 2001;69:704-11.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
ENG, Charis, MD, PhD	Professor / Principal Investigator

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Chicago	BA	1978-82	Biological Sciences
University of Chicago	PhD	1982-86	Developmental Bio
University of Chicago	MD	1982-88	Medicine
University of Cambridge	Postdoc	1992-95	Human Cancer Genetics

Positions and Honors

1988-91 Residency in Internal Medicine, Beth Israel Hospital, Boston, MA

1991-94 Clinical Fellowship, Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

1992-95 CRC Dana-Farber Fellowship in Human Cancer Genetics, University of Cambridge, UK

1992-95 Senior Registrar in Clinical Cancer Genetics, University of Cambridge Addenbrooke's Hospital, Cambridge, UK and Royal Marsden Hospital, London, UK

1994-95 Instructor in Medicine, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA

1995-98 Assistant Professor of Medicine, Dana-Farber Cancer Institute and Harvard Medical School, Boston

1995-98 Active Staff Physician, Adult Oncology, Dana-Farber Cancer Institute, Boston

1995-98 Associate Physician, Brigham and Women's Hospital, Boston

1998- North American Editor and Cancer Genetics Editor, *Journal of Medical Genetics*

1999-2002 Associate Professor (with tenure) of Medicine , The Ohio State University, Columbus

1999- Director, Clinical Cancer Genetics Program, James Cancer Hospital and Solove Research Institute, Comprehensive Cancer Center, Ohio State University, Columbus, OH

2001- Co-Director, Division of Human Genetics, Dept of Internal Medicine, The Ohio State University, Columbus

2000- William C. and Joan E. Davis Professor of Cancer Research, The Ohio State University, Columbus

2002- Professor (with tenure) of Medicine and Human Cancer Genetics, The Ohio State University, Columbus

1982 Phi Beta Kappa

1982 Sigma Xi Associate Membership and Sigma Xi Prize

1987 Sigma Xi Promotion to Full Membership

1988 Alpha Omega Alpha

1995 First Lawrence and Susan Marx Investigator in Human Cancer Genetics, Dana-Farber Cancer Institute

1999 American College of Physicians, Promotion to Fellowship

2001 American Society for Clinical Investigation, Elected Membership

2001- American Society for Clinical Oncology Advisory Committee on Genetic Testing for Cancer Susceptibility

Selected Peer Reviewed Publications (out of a total of 176 published and in press)

Eng C, Spechler SJ, Ruben R, Li FP. Familial Barrett esophagus and adenocarcinoma of the gastroesophageal junction. Cancer Epidemiol Biomark Prevent 1993; 2:397-9.

Nelen MR, Padberg GW, Peeters EAJ, 14 others, Ponder BAJ, Ropers HH, Kremer H, Longy M, **Eng C**. Localization of the gene for Cowden disease to 10q22-23. Nature Genet 1996; 13:114-6.

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Research Support

"Genetics of *PTEN* in Cowden syndrome and sporadic breast cancer"
PI: Charis Eng, MD, PhD
Agency: American Cancer Society Research Project Grant 10/1/98-9/30/01

The goal of this project is to examine the genotype-phenotype correlations of *PTEN* in Cowden syndrome and in sporadic breast cancers.

"Dissecting out the bifurcation of lipid and protein phosphatase activities in PTEN-mediated growth arrest in a breast cancer model"
PI: Charis Eng, MD, PhD
Agency: Susan G. Komen Breast Cancer Research Foundation Grant 10/1/00-9/30/02
The goal of this project is to determine the pathways downstream of PTEN's lipid and protein phosphatase activities as they related to breast carcinogenesis.

"A novel phosphatase gene on 10q23, *MINPP*, in hereditary and sporadic breast cancer"
PI: Charis Eng, MD, PhD
Agency: Department of Defense Idea Award 8/1/00-7/31/03
The goal of this project is to examine another phosphatase with overlapping function to PTEN as a non-PTEN susceptibility gene for Cowden syndrome and isolated breast cancer.

"Genetic analysis of the role of the microenvironment in epithelial tumor progression"
PI's: Charis Eng, MD, PhD, Gustavo Leone, PhD and Michael C. Ostrowski, PhD
Agency: V Foundation Jimmy V Golf Classic Research Award 3/1/01-2/28/04
The goal of this award is to provide seed moneys to gather preliminary data and make reagents related to tumor-microenvironmental interactions so that a group grant, eg PPG, may result from such work, as well as novel targets for therapy and prevention

"RET complex polymorphisms in Hirschsprung disease"
PI: Charis Eng, MD, PhD
Agency: National Institutes of HealthR01 Research Project Grant 7/1/01-6/30/05
The goal of this project is to identify and characterise common low penetrance alleles within *RET* and the genes which encode its ligands and co-ligands in "sporadic" medullary thyroid carcinoma as well as sporadic Hirschsprung disease

"Genetic alterations in the epithelial and stromal compartments of prostate adenocarcinomas"
PI: Charis Eng, MD, PhD
Agency: Department of Defense New Investigator Award 12/15/01-1/15/05
The goal of this study is to build a genome-wide genetic model of step-wise, multistage carcinogenesis in the prostate involving the epithelium and stroma.

"Genetics of *PTEN* in Cowden and related syndromes and familial cancer"
PI: Charis Eng, MD, PhD
Agency: American Cancer Society Research Scholar Grant 7/1/02-6/30/06
The goal of the project is to determine the individual-as-unit *PTEN* genotype-organ-specific phenotype risk of cancer in individuals with *PTEN* mutations, and to determine the risk and age of onset of each type of cancer.

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Germline Mutations in *BMPR1A/ALK3* Cause a Subset of Cases of Juvenile Polyposis Syndrome and of Cowden and Bannayan-Riley-Ruvalcaba Syndromes*

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Juvenile polyposis syndrome (JPS) is an inherited hamartomatous-polyposis syndrome with a risk for colon cancer. JPS is a clinical diagnosis by exclusion, and, before susceptibility genes were identified, JPS could easily be confused with other inherited hamartoma syndromes, such as Bannayan-Riley-Ruvalcaba syndrome (BRRS) and Cowden syndrome (CS). Germline mutations of *MADH4* (*SMAD4*) have been described in a variable number of probands with JPS. A series of familial and isolated European probands without *MADH4* mutations were analyzed for germline mutations in *BMPR1A*, a member of the transforming growth-factor β–receptor superfamily, upstream from the SMAD pathway. Overall, 10 (38%) probands were found to have germline *BMPR1A* mutations, 8 of which resulted in truncated receptors and 2 of which resulted in missense alterations (C124R and C376Y). Almost all available component tumors from mutation-positive cases showed loss of heterozygosity (LOH) in the *BMPR1A* region, whereas those from mutation-negative cases did not. One proband with CS/CS-like phenotype was also found to have a germline *BMPR1A* missense mutation (A338D). Thus, germline *BMPR1A* mutations cause a significant proportion of cases of JPS and might define a small subset of cases of CS/BRRS with specific colonic phenotype.

Introduction

The major hamartomatous-polyposis syndromes comprise juvenile polyposis syndrome (JPS [MIM 174900]), Peutz-Jeghers syndrome (PJS [MIM 175200]), Cowden syndrome (CS [MIM 158350]), and Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480]). Whereas CS and a subset of BRRS are allelic (Marsh et al. 1997*a*, 1999), current evidence suggests that CS and BRRS are genetically distinct from JPS and PJS (for reviews, see Eng and Ji 1998; Eng and Parsons 2001). PJS is an autosomal dominant disorder characterized by perioral pigmented spots, hamartomatous polyposis, and a risk for colon and breast cancers (Boardman et al. 1998; for review, see Eng et al. 2001). Germline mutations of the nuclear serine-threonine–kinase gene *LKB1/STK11* cause most cases of PJS (Hemminki et al. 1998; Jenne et al. 1998). CS is a poorly recognized autosomal dominant cancer syndrome characterized by multiple ha-

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Table 1

Clinical Features Present in Probands and Families with JPS

Proband or Family	Cancer		Anomalies		
	Colon	Other	Cardiac	Head/Facial	Other
BMPR1A-mutation positive:					
16[a]	Yes	No	No	No	No
18[a]	Yes	Esophageal	No	No	No
10[a]	Yes	No	No	No	No
19[a]	Yes	Unknown	No	No	No
FT[a]	Yes	No	No	Hypertelorism, macrocephaly	No
SM316	No	No	No	Hypertelorism, macrocephaly	No
VR	Unknown	No	No	No	Porphyria, malrotation of gut
1469	Unknown	Unknown	Unknown	No	No
JP7/19[a]	Yes	No	Ventricular septal defect, unspecified defects	No	No
JP8/1	Yes	Adrenal hamartoma, Wilms tumor	Ebstein anomaly	No	No
BMPR1A-mutation negative:					
MD[a]	Yes	No	No	Hypertelorism, macrocephaly	Telangiectasia
5[a]	Yes	Stomach	No	No	No
6[a]	Yes	No	No	Macrocephaly	No
1[a]	Yes	Small bowel	No	No	No
14[a]	Yes	No	No	No	No
22[a]	No	No	No	Macrocephaly? (diameter 56 cm [female])	Pigment naevi on trunk
CV	Unknown	No	Unknown	No	No
KS[a]	Unknown	No	No	No	No
YC[a]	Yes	No	No	No	No
SM106	No	No	Ventricular septal defect	Hypertelorism	No
12[a]	Yes	Small bowel	Aortic regurgitation	Subarachnoid hemorrhage	No
DM[a]	Yes	Stomach	Unknown	No	No
WN[a]	Yes	No	Unknown	Unknown	Unknown
JP1/1	Yes	Melanoma	No	No	Osler disease, epilepsy, empty sellar syndrome
JP2/13[a]	Yes	No	Unknown	Unknown	Unknown

Note.—By definition, all probands or families have at least one member with juvenile polyps.

[a] Familial.

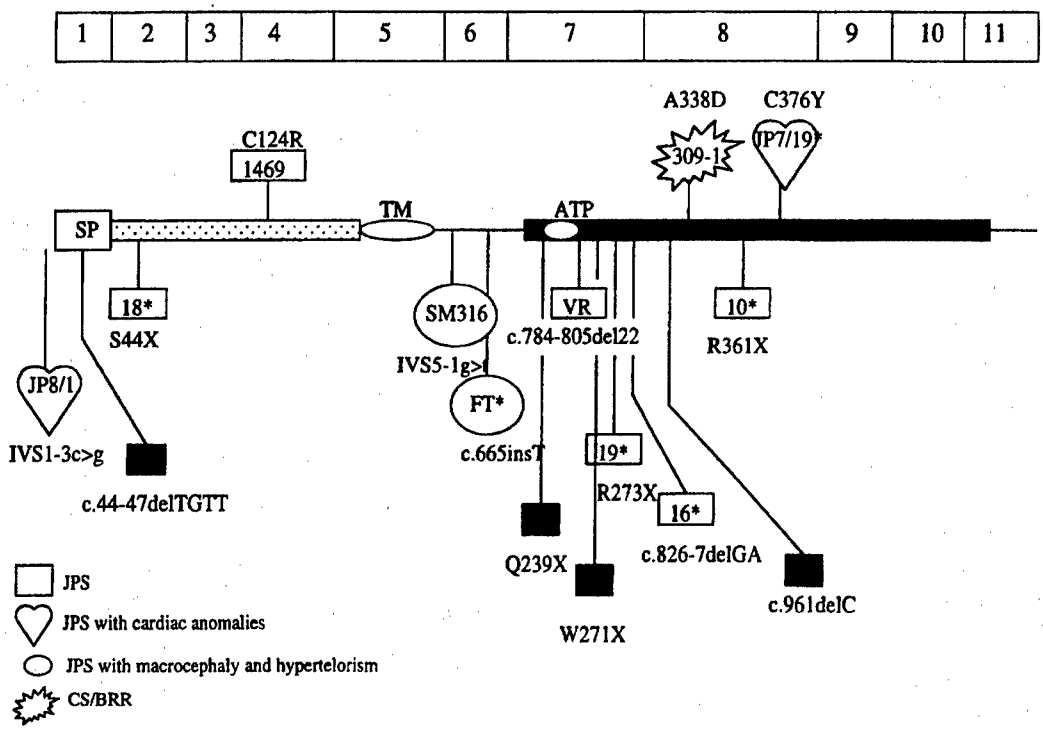

Figure 1 Spectrum of germline *BMPR1A* mutations in 14 probands with JPS and in 1 family with CS/BRRS. The exons are depicted by the numbered boxes at the top, and the domains of the receptor are depicted below. Signal peptide (SP), transmembrane domain (TM), ATP-binding domain (ATP), extracellular domain (*dotted bar*), and kinase domain (*black bar*) are shown. Black squares represent the four families' mutations published by Howe et al. (2001).

martomas and by a high risk for breast, thyroid, and endometrial cancers (Eng 2000). Although gastrointestinal hamartomatous polyposis can be documented if systematically searched for (Weber et al. 1998), the polyps are rarely symptomatic in CS, in contrast to the other three syndromes. BRRS is a congenital disorder characterized by macrocephaly, lipomatosis, thyroid problems, and pigmented macules on the glans penis in males (Gorlin et al. 1992); in BRRS, gastrointestinal hamartomatous polyposis can be quite prominent and symptomatic (Tsuchiya et al. 1998). Germline mutations in the tumor-suppressor gene *PTEN* cause 80% of cases of classic CS and 60% of cases of BRRS (Marsh et al. 1998*b*, 1999). There is little, if any, linkage evidence of genetic heterogeneity in CS (Nelen et al. 1996). The extent of genetic heterogeneity in BRRS is unknown. Clinical diagnosis of JPS is by exclusion, and JPS is characterized by gastrointestinal hamartomatous polyposis and by a risk for gastrointestinal cancers (for review, see Eng et al. 2001). Germline mutations in *MADH4* (*SMAD4*) have been described in a proportion of cases of JPS (Howe et al. 1998). From a nonsystematic survey of North American probands with JPS, it was estimated that ~35–60% of cases of JPS would harbor germline *MADH4* mutations (Howe et al. 1998); however, 3%–28% (weighted average 15%) of cases of JPS originating mainly from Europe have been found to carry *MADH4* mutations (Houlston et al. 1998; Friedl et al. 1999; Roth et al. 1999; Woodford-Richens et al. 2000*a*, and in press). Thus far, genes encoding several other SMADs have not been found to be associated with JPS (Bevan et al. 1999; Roth et al. 1999). Recently, germline truncating mutations in *BMPR1A/ALK3/SKR5* were described in four of four families segregating JPS (Howe et al. 2001). *BMPR1A*, on 10q21-q22, encodes a bone morphogenic-protein–receptor serine-threonine kinase that belongs to the transforming growth-factor β (TGFB)–receptor SMAD superfamily (for reviews, see Massagué 2000; Eng 2001). Members of the TGFB-receptor superfamily can homo-oligomerize or hetero-oligomerize.

We have examined *BMPR1A* for germline mutations, in a cohort of familial and sporadic cases of JPS, with the hypotheses that this mainly European cohort with a relatively low *MADH4*-mutation frequency would have a high frequency of *BMPR1A* mutations with a distinct mutational spectrum. Furthermore, because of the location of this gene in proximity to *PTEN* (Dahia et al. 2000) and, perhaps, because of its function, it also became a good candidate gene for susceptibility in *PTEN*-mutation–negative cases of CS and of BRRS.

Families, Material, and Methods

Families

Eighteen unrelated families with JPS and seven isolated cases of JPS were ascertained by clinical criteria described elsewhere (Marsh et al. 1997*b*) and were already known not to carry germline *MADH4* mutations. Although all families and individuals met the diagnostic criteria for JPS, some affected individuals had developed other tumors (table 1)—predominantly, colorectal adenomas and/or cancer—as is common in this condition (Woodford-Richens et al. 2000*a*). Twenty-one probands with CS/BRRS or CS/BRRS-like phenotype without germline *PTEN* mutations were ascertained by the revised operational diagnostic criteria of the International Cowden Consortium (Eng 2000) and by criteria described elsewhere (Marsh et al. 1998*a*, 1999). Probands and families with CS/BRRS-like phenotype have component features of CS/BRRS but do not meet the operational diagnostic criteria set forth by the International Cowden Consortium. All specimens were collected and analyzed, after informed consent was obtained, under protocols approved by each institution's Human Subjects Protection Committees.

Mutation Analysis

Genomic DNA was extracted from peripheral leukocytes, by standard protocols (Mathew et al. 1987). As template, 20–100 ng of DNA was used for 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, after a hot start) of PCR amplification of each of the 11 coding exons (thus, ALK3E3 corresponds to exon 1, etc.) and flanking intronic regions of *BMPR1A*, by use of the following primers: ALKE3F (5′-TCCAAAATTCAGTTGTATTCC-3′), ALKE3R (5′-CACATACATTACTAAAATGAACACTG-3′), ALKE4F (5′-GTCACGAAACAATGAGCTTT-3′), ALKE4R (5′-TTAAGAAGGGCTGCATAAAA-3′), ALKE5F (5′-CATTCAGACTCAAATTTCGTT-3′), ALKE5R (5′-TCTCATGGGTCCCAAATTA-3′), ALKE6F (5′-CCAAACCATTTCTAATTTTATCA-3′), ALKE6R (5′-CATGCTCCGACTTTTCTC-3′), ALKE7F (5′-CCAGGCTACCTAGAATTGAA-3′), ALKE7R (5′-AACAGCGGTTGACATCTAAT-3′), ALKE8F (5′-CCTCAAGGTTTTTCTTAGGG-3′), ALKE8R (5′-TCAACACACCATTCATGTCT-3′), ALKE9F (5′-TCATCAAGAGCTCAAACCTT-3′), ALKE9R (5′-ACCTCACTAGCCTTGTCAAA-3′), ALKE10F (5′-CCCTAGCCTATCTCTGATGA-3′), ALKE10R (5′-AACAGTGGGGCAAAGAAC-3′), ALKE11F (5′-TATTTTATTTTTGGCCCTCA-3′), ALKE11R (5′-TGATGAGTAAATCAACATAATCAG-3′), ALKE12F (5′-ATTTTTGTGCCCATGTTTT-3′), ALKE12R (5′-AATCACTTCTTCAGGGGACT-3′), ALKE13F (5′-ACTCAGTCCCCTGAAGAAGT-3′), and ALKE13R (5′-CTAGAGTTTCTCCTCCGATG-3′). The amplicons were gel- and column-purified and then were subjected to semiautomated PCR-based sequence analysis by an ABI-377a or a Perkin-Elmer 3700, as described elsewhere (Mutter et al. 2000).

Loss of Heterozygosity (LOH) Analysis

Available component tumors from *BMPR1A*-mutation–positive and *BMPR1A*-mutation–negative cases of JPS were subjected to LOH analysis with markers ALK3ca, ALK3ggaa, and D10S573, by techniques described elsewhere (Marsh et al. 1998*c*; Woodford-Richens et al. 2000*b*). Two component tumors from proband JP8/1 (table 1) was analyzed by sequencing of the amplicon containing the germline mutation, to examine allelic contribution.

Reverse-Transcriptase PCR (RT-PCR) Analysis

To assess the putative splice-site mutation in proband JP8/1, RNA was extracted from her component tumors, a Wilms tumor (table 1), and a colon carcinoma, and cDNA was synthesized. RT-PCR was performed using the primers 5′-GCATAGGTCAAAGCTGTTTGG-3′ and 5′-GCAAGGTATCCTCTGGTGCT-3′, with Ampli*Taq* Gold (Perkin-Elmer) at and annealing temperature of 60°C. Amplicons were fractionated through 2% agarose, were stained with ethidium bromide, and then were visualized with UV trans-illumination. Any aberrant bands noted on the gel were cut out of low-melting-point agarose, were gel- and column-purified, and then were subjected to sequence analysis.

Results

All 11 coding exons, splice junctions, and flanking intronic regions of *BMPR1A* were examined in 18 unrelated *MADH4*-mutation–negative families with JPS and in seven unrelated *MADH4*-mutation–negative individuals with isolated JPS. All available polyps from these cases showed no loss of SMAD4 expression. Overall, of 25 unrelated probands with JPS, 10 (40%) were found to have germline *BMPR1A* mutations (fig. 1): 6 (33%) of the 18 familial cases and 4 (57%) of the 7 isolated cases had germline mutations. In the mutation-positive familial cases in which samples from family members were available, the respective mutations were shown to segregate with affected status (data not shown)

Of the 10 germline *BMPR1A* mutations found in probands with JPS, all except 2 were nonsense, frameshift, or splice-site mutations predicted to result in truncated receptors (fig. 1). The missense mutations found in cases of JPS were examined in cohorts of 50 race-matched normal controls. None of the 100 normal control chromosomes were found to carry these missense mutations; furthermore, in the familial cases of JPS with C376Y, this mutation was found to segregate with dis-

ease. Loss of the wild-type allele in three component tumors—all of which were villous adenomas and two of which also had adenocarcinomatous components—from an affected family member was also demonstrated (fig. 2). The splice-site mutation IVS1-3c→g was shown to result in skipping of exon 1, and the component tumor (a Wilms tumor; table 1) from the proband had loss of the wild-type allele. A colorectal carcinoma from the proband with the IVS1 splice mutation did not show LOH. Thus, of five component tumors from *BMPR1A*-mutation–positive individuals, four were found to have loss of the wild-type allele. In contrast, 24 component tumors from 13 familial and isolated cases without germline *BMPR1A* mutations showed no LOH in that region (data not shown).

Although limited because of small sample size, genotype-phenotype associations were examined, especially those with respect to cardiac anomalies or to head/facial features (table 1 and fig. 1). Both among the 10 *BMPR1A*-mutation–positive families and among the 15 *BMPR1A*-mutation–negative families, 2 had cardiac anomalies; because of the limited size of sample, these were not considered statistically different. Similarly, there appeared to be no difference between the numbers of mutation-positive and mutation-negative families and individuals with macrocephaly or hypertelorism. Although there were only two probands/families with mutations as well as with the clinical features of hypertelorism and macrocephaly, both of these mutations—IVS5-1g→t (SM316) and c.665insT (FT)—occurred in the juxtatransmembrane domain (fig. 1).

Of 21 unrelated probands with CS/BRRS, without germline *PTEN* mutations, 1 was found to have a germline missense mutation, A338D, in exon 8 of *BMPR1A*. This missense alteration was not observed among 172 race-matched, geographically matched control chromosomes. Interestingly, the proband had only colonic polyposis, which comprised hamartomatous and adenomatous polyps and began at the age of 16 years, and lipomas. Her family history, however, comprised individuals with breast cancer, with renal-cell carcinoma, with brain tumor(s), and with melanoma. Taken together, these features constitute the minimum criteria (i.e., one major and three minor) for the diagnosis of CS (Eng 2000). It is acknowledged that the diagnosis of CS in this family barely met the minimum International Cowden Consortium diagnostic criteria, and some clinicians might consider this family to have a CS-like phenotype. None of the other probands with CS/BRRS or CS/BRRS-like phenotype were found to have *BMPR1A* mutations.

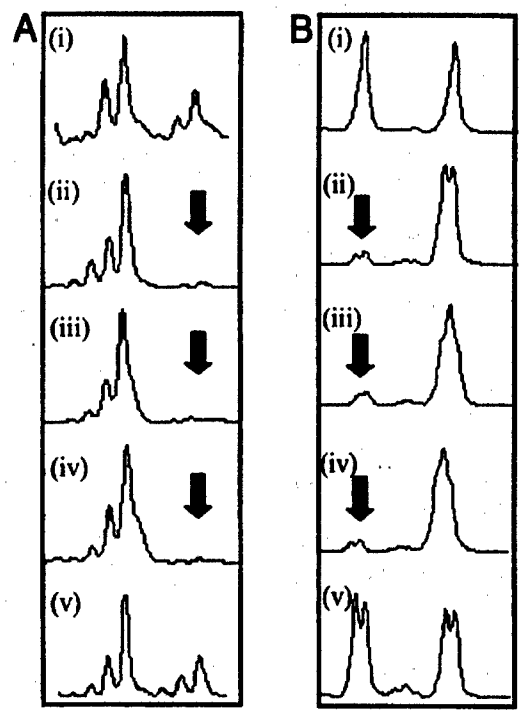

Figure 2 LOH analysis with microsatellite markers alk3ca (*A*) and alk3ggaa (*B*), which lie in proximity to *BMPR1A* (see text), and genomic DNA templates from family JP7/19, whose members harbor a germline missense mutation, generated from peripheral blood leukocytes (*i*), from villous adenoma (*ii*), from two villous adenomas with adenocarcinomatous components (*iii* and *iv*), and from normal tissue originating from the same archival section as one of the villous adenomas with adenocarcinoma (*v*).

Discussion

In this cohort of familial and isolated cases of JPS who are *MADH4*-mutation–negative and who originate mainly from Europe, 40% have been found to harbor germline *BMPR1A/ALK3* mutations. Thus, among European cases of JPS, *MADH4* mutations account for ≤28% of cases and *BMPR1A* mutations account for 40%. No systematic survey of cases of JPS originating in the United States has been performed yet, and it thus is unknown what proportion cases of JPS is due to *BMPR1A* mutations. Nonetheless, at least one other JPS-susceptibility gene should exist.

Overall, to date, 14 different germline *BMPR1A* mutations have been described in probands with JPS—10 in patients from this study and the 4 in U.S. kindreds described elsewhere (Howe et al. 2001). Of these germline *BMPR1A* mutations, 9 (64%) are located within exons 6–8 (exon 1 is the first coding exon; there are two other noncoding exons 5′ of exon 1), encoding part of the intracellular domain of the receptor (fig. 1), and, of these 9 mutations, 8 have occurred in the N-terminal 142 amino acids of the kinase domain, half of which are in close proximity to the ATP-binding site. There are no mutations located in or beyond the C-terminal half of the kinase domain. Interestingly, the two pro-

bands with mutations occurring in exons 5 and 6 both have macrocephaly and hypertelorism.

All but one of the nine mutations in the cytoplasmic domain are predicted to result in truncated receptors (fig. 1). The truncations all leave an intact transmembrane domain, such that the mutant receptors could be processed, to reach the plasma membrane, but are lacking all or part of the kinase domain. If the mutations in the cytoplasmic domain do result in truncated receptors, then these truncated receptors might be expected to bind ligand, but no signaling could occur. Thus, these intracellular-domain mutations might be predicted to act via dominant negative mechanisms. Family JP7/19 has a missense mutation in the middle of the kinase domain, C376Y. Residue 376 lies within the kinase domain, in close proximity to the active site, and is highly conserved among species—from *Caenorhabditis elegans* to mouse and rat. Four of the five mutations in the extracellular domain are predicted to result in truncated receptors. However, unlike the truncations in the cytoplasmic domain, two of the truncations would result in the lack of all or part of the signal peptide. The third truncation, S44X, results in a very short peptide without a transmembrane domain. Cysteine 124 lies in the cysteine-rich domain, which characterizes receptor kinases and is highly conserved across the TGFB family of type I and type II receptors, as well as across species (Kirsch et al. 2000). The ectodomain of BMPR1A has six intramolecular disulfide bridges between pairs of cysteines, which conformationally allows for BMP2 binding (Kirsch et al. 2000). Cysteine 124 is part of disulfide bond 4, and between the two cysteines forming this disulfide bridge lie nine key residues, which form part of the ligand-binding epitope. Loss of the sulfhydryl group at residue 124, as would be the case for this mutation, would therefore result in severe conformational alterations and in loss of the ability to bind ligand. The splice mutation IVS5-1g→t would be predicted to result in a receptor without a transmembrane domain. Thus, in general, extracellular-domain germline mutations—whether truncating or missense—together with the somatic second hit—as evidenced by LOH in the *BMPR1A* region in the majority of component tumors, both benign and malignant—might result in physical or functional lack of receptor. These observations contrast with those of Howe et al. (2001), who failed to detect LOH in component tumors from mutation-positive families. Our data demonstrating that *BMPR1A* behaves in accordance with the Knudson two-hit theory strongly suggest that *BMPR1A* encodes a tumor suppressor and likely also plays a gatekeeping function (Kinzler and Vogelstein 1998), much like SMAD4 itself (Woodford-Richens et al. 2000*b*).

Although the sample size is small, it would appear that, among the nine cytoplasmic-domain mutations, seven have occurred in familial cases of JPS whereas only two have occurred in isolated cases of JPS. In contrast, of the four extracellular-domain mutations, two occur in familial cases and three occur in isolated cases. Because of the small sample sizes of each subset, no statistical significance can be inferred. However, an interesting hypothesis to test in the future is that *BMPR1A* mutations that occur in the cytoplasmic domain and that are predicted to be dominant negative are associated with higher penetrance and with familial transmission. Because we have demonstrated LOH in component tumors from mutation-positive individuals—and if this hypothesis is correct—then the dominant-negative effect must act against other TGFB-receptor–family partners with which BMPR1A normally hetero-oligomerizes. Extracellular mutations that mainly result in haploinsufficiency, on the other hand, are associated equally with isolated and familial cases.

Because CS and BRRS lie within a single spectrum (Marsh et al. 1999), we chose to examine probands with CS/BRRS and CS/BRRS-like phenotype as one group. Only one such proband with CS/CS-like phenotype was found to harbor a *BMPR1A* mutation—specifically, A338D. This missense mutation occurs in the kinase domain—more specifically, immediately downstream of the kinase catalytic core—and in a residue that is highly conserved across species, from *C. elegans* to mouse and rat. Thus, if an acidic hydrophilic residue (aspartate) were substituted for a neutral nonpolar residue (alanine), the kinase catalytic core would be predicted to be disrupted. Although ligand binding might still be possible, this mutation could be predicted to result either in a loss of substrate specificity or in a receptor that might not be able to bind substrate.

Despite some initial confusion that germline *PTEN* mutations might be associated with rare cases of JPS (Olschwang et al. 1998), over the course of the past 4 years of clinical and molecular-epidemiologic analyses, it has become obvious that the presence of germline *PTEN* mutations defines CS and BRRS, regardless of the manner of clinical presentation (Eng and Ji 1998; Kurose et al. 1999; Marsh et al. 1999). This is germane for clinical cancer genetic practice, because the presence of *PTEN* mutations implies organ-specific surveillance of the patient and of his or her family. On the other hand, detection of a *MADH4* or a *BMPR1A* mutation should be considered diagnostic of JPS. In our opinion, families with CS/BRRS or CS/BRRS-like phenotype with *BMPR1A* mutations must therefore, on the basis of molecular data, be reclassified as having JPS.

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Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for JPS [MIM 174900], PJS [MIM 175200], CS [MIM 158350], and BRRS [MIM 153480])

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Genetic model of multi-step breast carcinogenesis involving the epithelium and stroma: clues to tumour–microenvironment interactions

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ABSTRACT

Although numerous studies have reported that high frequencies of loss of heterozygosity (LOH) at various chromosomal arms have been identified in breast cancer, differential LOH in the neoplastic epithelial and surrounding stromal compartments has not been well examined. Using laser capture microdissection, which enables separation of neoplastic epithelium from surrounding stroma, we microdissected each compartment of 41 sporadic invasive adenocarcinomas of the breast. Frequent LOH was identified in both neoplastic epithelial and/or stromal compartments, ranging from 25 to 69% in the neoplastic epithelial cells, and from 17 to 61% in the surrounding stromal cells, respectively. The great majority of markers showed a higher frequency of LOH in the neoplastic epithelial compartment than in the stroma, suggesting that LOH in neoplastic epithelial cells might precede LOH in surrounding stromal cells. Furthermore, we sought to examine pair-wise associations of particular genetic alterations in either epithelial or stromal compartments. Seventeen pairs of markers showed statistically significant associations. We also propose a genetic model of multi-step carcinogenesis for the breast involving the epithelial and stromal compartments and note that genetic alterations occur in the epithelial compartments as the earlier steps followed by LOH in the stromal compartments. Our study strongly suggests that interactions between breast epithelial and stromal compartments might play a critical role in breast carcinogenesis and several genetic alterations in both epithelial and stromal compartments are required for breast tumour growth and progression.

INTRODUCTION

Breast cancer is the most common and second most lethal cancer in women in Western countries. Numerous studies have focused on the role of chromosome abnormalities and gene mutations in sporadic breast cancer, but to date no clear model of the critical events or delineation of primary abnormalities has emerged. Various chromosome arms have been observed to be affected by a high frequency of structural or numerical abnormalities (1–5). Although several of these chromosome arms appear to be the sites of putative tumour suppressor genes (TSGs), the number and identity of TSGs relevant for mammary carcinogenesis is unknown. At the molecular level, several somatic mutations in genes residing in these regions have been described (1,6–9). Despite this abundance of data, the relevance, role and timing of most of the described genetic abnormalities in sporadic breast cancer are still unclear. It is also not known whether specific mutations play relevant roles as causative factors or are the consequence of the general genomic instability and progression in breast tumours.

A few studies have previously demonstrated that loss of heterozygosity (LOH) identified at various chromosomal loci at high frequency in invasive cancer is already present in *in situ* carcinoma, atypical ductal hyperplasia, non-atypical hyperplasia of the breast, and perhaps adjacent normal epithelial cells, although these studies predated laser capture microdissection (LCM), hence, contamination from clearly malignant tissue cannot be excluded (10–15). These observations are also found in colonic adenomas (16), Barrett oesophageal metaplasia

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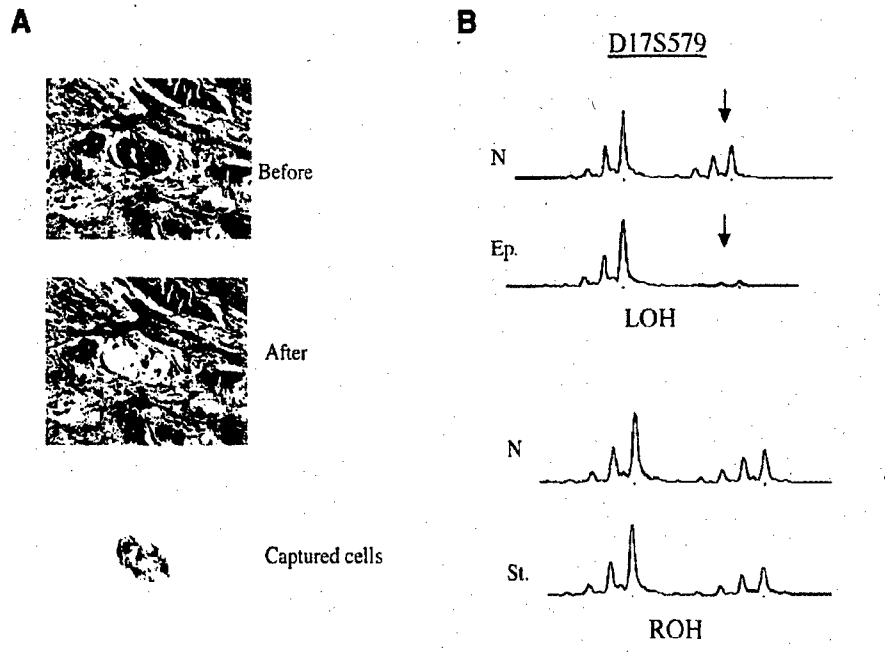

Figure 1. (**A**) LCM from breast cancer specimen. Captured cells are the neoplastic epithelial component. The surrounding stromal fibroblasts are immediately adjacent to the removed cells (arrow). (**B**) Illustrative examples of LOH (arrows) and retention of heterozygosity (ROH) at D17S579. N, normal cells; Ep., epithelial cells; St., stromal cells.

(17,18) and lung hyperplasias (19). These reports indicate that the majority of premalignant or precursor lesions share their LOH phenotypes with invasive disease in the same organs, providing novel biologic evidence that they are genetically and perhaps evolutionarily related. Nonetheless, until recently, any cancer such as breast cancer was treated as a single amorphous entity. Most such genetic studies were uniformly performed on the entire tumour without regard to its components, despite the fact that a few groups were quite aware of both epithelial and stromal components of tumours, and the cell biology of the tumour 'microenvironment' has been described for the last 20 years. Thus, until now, when a genetic alteration, be it intragenic mutation, regional amplification or deletion manifested by LOH, is attributed to a breast cancer, it is unclear if the alteration is actually occurring in the epithelial compartment, the surrounding stromal compartment or both.

The effects of the metabolism of the tumour stroma, locally as well as systemically, are largely unknown. Although the stroma has generally been considered a silent bystander during epithelial carcinogenesis, the concept that the microenvironment is central to maintenance of cellular function and tissue integrity provides the rationale for the idea that its disruption can contribute to neoplasia (20). Several studies performed *in vivo* and *in vitro* indicated that the growth and invasive potentials of carcinoma cells are influenced through interactions with host stromal cells (21–23). Despite these progressive cell biological studies, the precise genetic mechanisms leading to tumour progression remain unclear. Even less clear is the role of differential genetic alterations in the epithelial neoplastic component and its surrounding stroma.

Recently, Moinfar *et al.* (5) reported the high frequency of LOH in the mammary stroma with breast cancer. They examined 11 patients with ductal carcinoma *in situ*, including five cases with invasive ductal carcinoma, and found LOH in the stromal cells. Although these investigators identified frequent genetic alterations in the mammary stroma, each component of the breast carcinoma was manually microdissected; thus cross-contamination cannot be excluded. Further, they examined a limited number of samples and a limited number of microsatellite markers on only five chromosome arms. In this present study, we sought to systematically examine for genetic alterations in the epithelial and stromal components of invasive adenocarcinomas of the breast with the DNA extracted from cells from each compartment obtained by LCM. We found frequent LOH in both epithelial and/or stromal components of breast cancer and identified associations among LOH at various chromosomal regions, suggesting that genetic alterations in the epithelial and surrounding stromal cells are involved in the breast tumorigenesis through concurrent and independent pathways.

RESULTS

LCM of each specimen was performed to selectively obtain normal epithelial or stromal cells, carcinomatous epithelial cells and stromal cells surrounding the epithelial carcinoma (e.g. Fig. 1A). LOH at each of the 13 chromosomal regions was detected in epithelial and/or stromal compartments among the 41 invasive adenocarcinomas of the breast (e.g. Fig. 1B). Table 1 summarizes the frequencies of LOH observed at 13 loci in neoplastic epithelial and surrounding stromal cells. Among the 13 microsatellite markers examined, the LOH frequency ranged from 25% (9/36) at D3S1581 (3p14–q21) to 69% (22/32) at D17S796 (17p13) in the neoplastic epithelial compartment, and from 17% (6/36) also at D3S1581 to 61% (20/33) at D2S156 (2q34) in the surrounding stromal

Table 1. LOH frequencies and distribution in the epithelial and stromal cells

Chromosomal region	Marker	Cases with LOH in Ep[a]/informative cases	Cases with LOH in St[b]/informative cases	Cases with LOH in either Ep or St/ informative cases	Cases with LOH only in Ep	Cases with LOH only in St	Cases with LOH in both Ep and St	McNemar's test *P*-value (exact *P*-value)
1p36	D1S228	16/34 (47%)	10/34 (29%)	21/34 (62%)	11	5	5	0.1336 (0.2101)
2q34	D2S156	16/33 (48%)	20/33 (61%)	26/33 (79%)	6	10	10	0.3173 (0.4545)
3p14.2–p21.2	D3S1581	9/36 (25%)	6/36 (17%)	11/36 (31%)	5	2	4	0.2568 (0.4531)
3p24.3–p25.1	D3S1286	17/37 (46%)	14/37 (38%)	23/37 (62%)	9	6	8	0.4386 (0.6072)
6q25.3	D6S437	14/29 (48%)	15/29 (52%)	20/29 (69%)	5	6	9	0.7630 (1.000)
8p23.2	D8S264	21/32 (66%)	18/32 (56%)	25/32 (78%)	7	4	14	0.3657 (0.5488)
10q23.3	D10S1765	15/36 (42%)	10/32 (31%)	16/32 (50%)	6	5	5	0.7630 (1.000)
11q23	D11S912	19/37 (51%)	16/37 (43%)	25/37 (68%)	9	6	10	0.4386 (0.6072)
13q14	D13S155	18/33 (55%)	16/33 (48%)	23/33 (70%)	7	5	11	0.5637 (0.7744)
16q24.3	D16S413	20/31 (65%)	13/32 (41%)	23/31 (74%)	11	3	9	0.0325 (0.0574)
17p13	D17S796	22/32 (69%)	14/32 (44%)	24/32 (75%)	10	2	12	0.0209 (0.0386)
17q21	D17S579	17/32 (53%)	11/32 (34%)	21/32 (66%)	10	4	7	0.1088 (0.1796)
22q12.2–q13.1	D22S277	15/37 (41%)	15/37 (41%)	21/37 (57%)	6	6	9	1.000 (1.000)

[a]Ep, epithelium; [b]St, stroma.

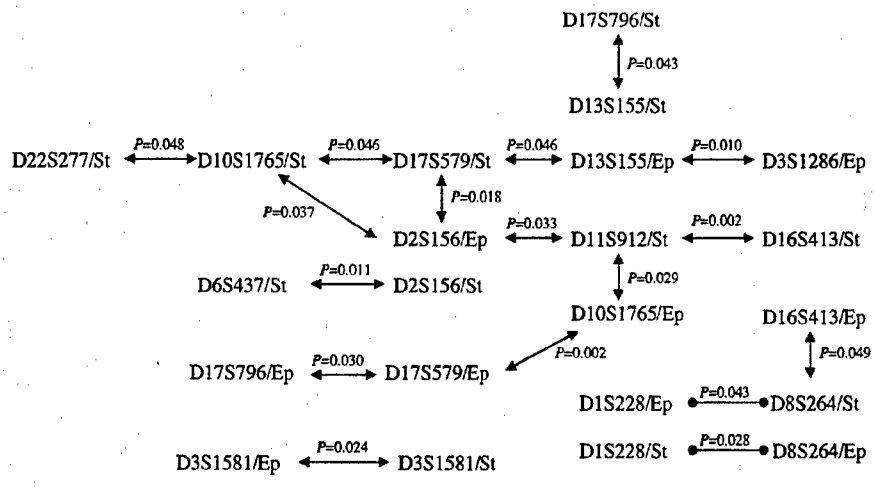

Figure 2. Positive and negative associations between markers in epithelial and stromal compartments of adenocarcinomas of the breast. Double-headed arrows denote positive correlations while double-knobbed lines denote negative correlations. Numbers above the arrows or lines are *P*-values (Fisher's exact test). Ep, epithelial cells; St, stromal cells.

component (Table 1). D2S156 (2q34) and perhaps D6S437 (6q25) were the only two markers demonstrating a higher frequency of LOH in the surrounding stromal compartment compared with the neoplastic epithelial cells. In contrast, the great majority of markers showed a higher frequency of LOH in the neoplastic epithelial compartment compared with the surrounding stromal cells (Table 1).

On further inspection of the differential LOH data, it can be noted that for certain markers, LOH predominates in the neoplastic epithelial compartment, for others, LOH predominates in the stromal compartment, and for yet other markers, it occurs in both compartments (Table 1). For instance, LOH at 16q24.3 (D16S413) was identified more frequently in only the neoplastic epithelial cells (11 tumours) than in only the stromal cells (three tumours; $P = 0.0325$, McNemar's test). The number of tumours that showed LOH at 17p13 (D17S796) only in stromal cells (two tumours) was significantly fewer than that having LOH at 17p13 in both epithelial and stromal cells or only in the neoplastic epithelial cells (12 or 10 tumours; $P = 0.0209$, McNemar's test).

We then looked for pair-wise associations of dependency or independency of particular genetic alterations with one another in either epithelial or stromal compartments (Fig. 2). We found that there were statistically significant associations in 17 pairs

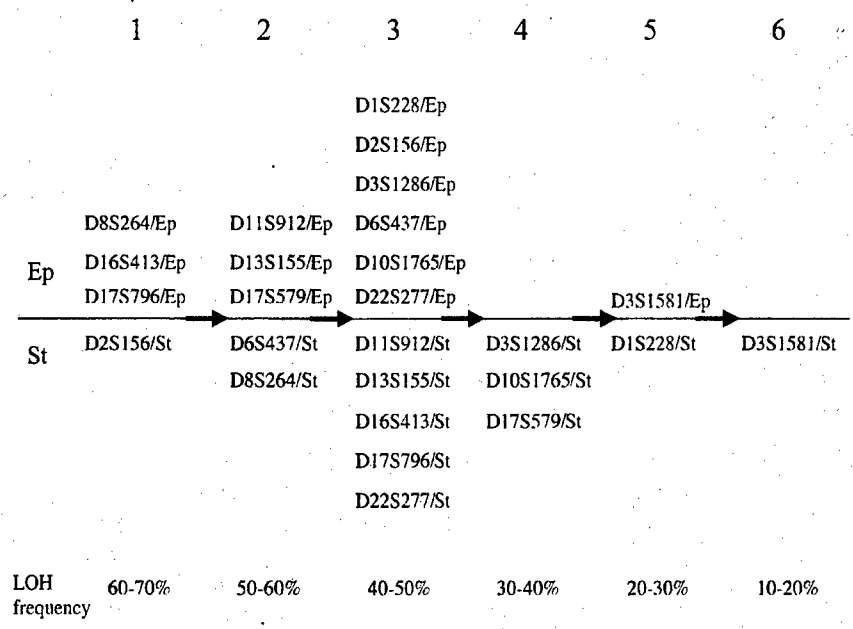

Figure 3. Proposed genetic model of multistage, stepwise carcinogenesis in the breast involving the epithelial and stromal compartments. Ep, epithelial cells; St, stromal cells.

(17/325; 5.3%) of markers (Fig. 2). Fifteen pairs (15/17; 88%) showed positive associations and two pairs (2/17; 12%) showed negative associations (Fig. 2). LOH at four markers, D10S1765, D11S912 and D17S579 in the surrounding stromal compartment and D2S156 in the neoplastic epithelial compartment, were associated with three or more sites of LOH (Fig. 2). Of note, LOH at D10S1765 in the stromal compartment was associated with LOH at D17S579 and D22S277 in the stromal compartments and also with LOH at D2S156 in the neoplastic epithelial compartments. LOH at D17S579 in the stromal compartment, in turn, was found to be associated with LOH at D2S156 and D13S155 in the neoplastic epithelial compartment and at D10S1765 in the surrounding stromal compartment. In contrast, LOH at D8S264 (8p23.2) in the neoplastic epithelial cells is negatively correlated with LOH at D1S228 (1p36) in the surrounding stromal cells. Interestingly, LOH at D8S264 in the surrounding stromal compartment is negatively correlated with LOH at D1S228 in the neoplastic epithelial compartment.

DISCUSSION

In this present study, we have found frequent LOH in both neoplastic epithelial and surrounding stromal cells of invasive adenocarcinomas of the breast. In our series of 41 breast cancer samples, we identified LOH in the neoplastic epithelial compartment, ranging from 25% (9/36) to 69% (22/32), and in the stromal compartment, ranging from 17% (6/36) to 61% (20/33), respectively. Of note, the great majority of markers demonstrated a higher frequency of LOH in the neoplastic epithelial compartment compared with the surrounding stromal cells (Table 1). Further inspection of the differential LOH data indicated that the frequency of LOH only in the stromal compartment occurred among fewer tumours than that in both neoplastic epithelial and surrounding stromal compartments, or that in only the neoplastic epithelial cells (Table 1). In the field of human cancer genetics, it has been shown that markers with the highest frequency of LOH represent those with the earliest genetic alterations, the so-called first 'hits', and the one with the lowest frequency of LOH represents the latest 'hit' in carcinogenesis (16). Thus, under this assumption, from our data, we can propose a genetic model of multistage, stepwise carcinogenesis in the breast, according to the relative frequencies of LOH in the epithelial and stromal compartments (Fig. 3). Our proposed model encompasses data that shows a higher frequency of LOH in breast epithelial cells occurs earlier than in the stromal compartment (Fig. 3). Although Moinfar *et al.* (5) worked without the advantage of LCM and used markers representing only five chromosomal regions and a sample size of 11, their observations of relative frequencies of LOH in the neoplastic epithelium (occurring in 1/4 to 3/3 tumours) compared with presumably surrounding stroma (occurring in 1/4 to 4/5 tumours) might be interpreted as concurrent with ours. These data together with our observations suggest that genetic alterations in the epithelial compartment, at least in some chromosomal regions, precede the genetic changes in the surrounding stromal cells. If in fact we may extrapolate that each region of LOH represents at least one putative TSG, then it is possible that the same putative gene involved in epithelial carcinogenesis plays some role in the stroma at a later stage, with the possible exception of D2S156. In our study, the earliest genetic alterations occurred at D8S264, D16S413 and D17S796 in the epithelium as well as D2S156 in the stroma (Fig. 3). It is almost certain that the D17S796 marker represents *TP53*, and indeed, *TP53* alterations have been noted amongst the most frequent and earliest somatic alterations in prior studies involving 'whole' breast carcinomas (24,25). The regions of D8S264 and D16S413 have yet to yield convincing TSGs involved in breast carcinogenesis. Our data would strongly support the existence of one or more TSGs residing in these two regions which when

mutated participate in the initiation of cancer within the breast epithelium. Of interest, LOH at D2S156 in the stromal cells is also scored as an early event (Fig. 3). This has not been a region noted to have LOH in whole breast cancers. Nonetheless, our data suggest that there will be at least one important gene residing in that interval which plays a prominent role in the initiation of breast carcinogenesis, possibly from a microenvironmental or 'landscaper' point of view (26). The genetic model proposed (Fig. 3) assumes for simplicity that frequency of occurrence reflects temporal sequence. This in turn assumes an equivalent effect on tumorigenesis and early progression between all alterations. However, it is recognized that an alternative possibility is that some alterations may be dominant while others may require the coqperation of parallel or multiple complex alterations at other sites to facilitate progression, and that this would influence the prevalence of the alteration in advanced tumours. In the case of these different assumptions, a lower frequency of stromal alterations could reflect the fact that stromal alterations are not dominant and only exert an indirect effect on the adjacent epithelium, or only exert an effect in collaboration with others, to influence the overall process of tumorigenesis.

The apparent asynchronous LOH at each marker between epithelium and stroma might suggest that while the neoplastic epithelium is clonal, as is the stroma, these observations may support one viewpoint that epithelium and stroma derive from different cellular origins. However, there are advocates of a common cellular origin of both epithelium and stroma (27). If this latter is true, then in the context of our observations, the LOH in epithelium and stroma occurred after the divergence of epithelial and stromal cell from the presumed common cell.

Despite a relatively small sample size, we were able to examine pair-wise associations between regions and compartments where LOH occurred (Fig. 2). For example, LOH at D17S579 in the neoplastic epithelial cells was associated with LOH at D17S796 and D10S1765 in the neoplastic epithelial compartments. These chromosomal loci contain several putative TSGs. The polymorphic markers, D17S579 (17q21), D17S796 (17p13) and D10S1765 (10q23.3), are in proximity to the *BRCA1*, *TP53* and *PTEN* genes, respectively. Previous reports have identified that there are significant associations between LOH of *BRCA1* and *TP53* (28,29) or *PTEN* gene (28) in sporadic heterogeneous breast cancer samples. Crook *et al.* (30) found a high proportion of 'whole' breast and ovarian tumours from *BRCA1* mutation carriers had *TP53* mutations. Our proposed genetic model does suggest that LOH at D17S796 in the neoplastic epithelial cells is the earliest hit, LOH at D17S579 in the neoplastic epithelial cells is the second one, then LOH at D10S1765 in the neoplastic epithelial cells is the third hit in these consequences (Table 1 and Fig. 3). The association between LOH of *BRCA1* and *PTEN* is, therefore, one of the genetic alterations that might be expected to occur as a consequence of the loss of *BRCA1* and *TP53*. LOH at D2S156 in the neoplastic epithelial compartment was associated with LOH at three markers, D10S1765, D11S912 and D17S579, in the surrounding stromal compartments. The reciprocal interaction between epithelial and stromal cells plays a key role in the morphogenesis, proliferation and differentiation of epithelial cells (31–33). Most of the intercellular substances, extracellular matrix (ECM) molecules that are required for tumour growth and progression are produced by the stromal cells (34). It is well demonstrated that altered gene expression occurs between normal and neoplastic breast stroma (35) and that stromal cells play a critical role in the production and possible dissolution of the ECM (36,37). Thus, genetic alterations in the stromal cells may change the interaction between epithelial cells and ECM molecules and influence the tumour invasion and dissemination (22,23). Our results suggest that LOH at 2q34 (D2S156) might precede LOH of three chromosomal loci in the stromal compartments (Figs 2 and 3). Therefore, we hypothesized that loss of a putative TSG on 2q34 might play an important role in genetic alterations of stromal compartments, which in turn might influence tumour invasion and dissemination through ECM remodelling. If this hypothesis is correct, then we can further hypothesize that genetic alterations in the stroma might predict for poorer prognosis due to increased tumour invasiveness.

In summary, we have found frequent LOH in both neoplastic epithelial and surrounding stromal compartments in invasive adenocarcinomas of the breast and statistically significant associations among the LOH at various chromosomal regions. We also propose a multi-step genetic model of breast carcinogenesis involving epithelium and stroma, which can help build further hypotheses and guide future studies of reciprocal interactions between breast neoplastic epithelial and stromal cells in tumour initiation, progression, invasion and metastases. Such studies might eventually lead to novel therapeutic strategies, which selectively target epithelium or stroma.

MATERIALS AND METHODS

Breast cancer samples

Forty-one archival (formalin-fixed and paraffin-embedded) tissues that were distinct cases of clinically sporadic primary invasive adenocarcinomas of the breast were used. Twenty-six samples were obtained from the National Cancer Institute of Canada Manitoba Breast Tumour Bank and 15 were from the Department of Pathology of The Ohio State University.

Microdissection of tissue and DNA extraction

Microdissection of carcinomatous epithelial cells, surrounding stromal cells, and normal epithelial or stromal cells from fixed, paraffin-embedded sections of breast was performed using an Arcturus PixCell II Laser Capture microdissecting microscope (Arcturus Engineering Inc., Mountain View, CA). This system utilizes a transparent thermoplastic film applied to the surface of the tissue section on standard histopathology slides. The breast cancer epithelial, surrounding stromal, and normal epithelial or stromal cells to be microdissected were identified and targeted through a microscope, and a narrow (~15 uM) carbon dioxide laser-beam pulse specificity activated the film above these cells. The resulting strong focal adhesion allowed selective procurement of only the targeted cells (38) (Fig. 1A). The cells removed in Figure 1A are the neoplastic epithelial component. The surrounding stromal fibroblasts are immediately adjacent to the removed cells (Fig. 1A, arrow). It is acknowledged that while LCM minimizes cross-contamination of cell types, it does not guarantee against it. However, the very 'clean' LOH (virtually all or none) which we have obtained does suggest that any cross-contamination is not significant.

DNA from microdissected tissue was extracted in 50 µl of solution containing 0.04% proteinase K, 1% Tween-20, 10 mM Tris–HCl (pH 8.0), and 1 mM EDTA at 37°C overnight followed by heat inactivation at 95°C for 10 min.

LOH analysis

For purposes of this study, genomic DNA, extracted from paraffin embedded tissues, served as template for PCR amplification of 13 microsatellite markers selected from a comprehensive genetic map of the human genome (39). Fluorescent-labelled polymorphic markers, including D1S228 (1p36), D2S156 (2q34), D3S1581 (3p14.2–p21.2), D3S1286 (3p24.3–p25.1), D6S437 (6q25.3), D8S264 (8p23.2), D10S1765 (10q23.3), D11S912 (11q23), D13S155 (13q14), D16S413 (16q24.3), D17S796 (17p13), D17S579 (17q21) and D22S277 (22q12.2–q13.1), were used for this analysis. All subsequent PCRs were carried out using 0.5 µM each of forward and reverse primer in 1× PCR buffer (Qiagen, Valencia, CA), 1.5 mM $MgCl_2$ (Qiagen), 1× Q-buffer (Qiagen), 1.25 U of HotStar Taq polymerase (Qiagen) and 200 µM of each dNTP (Gibco, Gaithersburg, MD) in a final volume of 25 µl. After a denaturation at 95°C for 14 min, reactions were subjected to 40 cycles of 94°C for 1 min, 55–60°C for 1 min, and 72°C for 1 min followed by 10 min at 72°C. PCR reactions and genotyping were repeated at least a second time to confirm the data. Amplified PCR products were separated by electrophoresis through 6% denaturing polyacrylamide gels, and the signal was detected with an Applied Biosystems model 377×l semi-automated DNA sequencer (Applied Biosystems, Perkin-Elmer Corp., Norwalk, CT). The results were analysed by automated fluorescence detection using the GeneScan collection and analysis software (GeneScan, Applied Biosystems). Scoring of LOH was initially performed by inspection of the GeneScan analysis output. A conservative ratio of peak heights of alleles between germline DNA and somatic DNA ≥1.9:1 were used to define LOH in this study (40).

Statistical analysis

Comparisons for statistical significance were performed by using either the standard Fisher's exact test (2-tailed) or the McNemar's test for matched pairs at the $P = 0.05$ level of significance. McNemar's test was used when interest focused on differences in proportions of patients with LOH in either stromal or epithelial cells, but not both. This test determines whether, in these cases of discordance, there are a disproportionate number of patients with LOH in one of the two sites. McNemar's test is used in recognition of the fact that the stromal and epithelial cells are taken from the same breast tissue and, hence, are matched. However, the Fisher's exact test was also employed because it is unclear from a biological point of view whether each data point (i.e. LOH at any one marker) is dependent on the next (i.e. LOH at other markers).

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